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Soria-Lozano *et al. BMC Microbiology* (2015) 15:187
DOI 10.1186/s12866-015-0524-3

RESEARCH ARTICLE

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In vitro effect photodynamic therapy with differents photosensitizers on cariogenic microorganisms

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Abstract

Background: Antimicrobial photodynamic therapy has been proposed as an alternative to suppress subgingival species. This results from the balance among *Streptococcus sanguis*, *Streptococcus mutans* and *Candida albicans* in the dental biofilm. Not all the photosensitizers have the same photodynamic effect against the different microorganims. The objective of this study is to compare in vitro the photodynamic effect of methylene blue (MB), rose Bengal (RB) and curcumin (CUR) in combination with white light on the cariogenic microorganism *S. mutans, S. sanguis* and *C. albicans*.

Results: Photodynamic therapy with MB, RB and CUR inhibited 6 log 10 the growth of both bacteria but at different concentrations: 0.31–0.62 μg/ml and 0.62–1.25 μg/ml RB were needed to photoinactivate *S. mutans* and *S. sanguis*, respectively; 1.25–2.5 μg/ml MB for both species; whereas higher CUR concentrations (80–160 μg/ml and 160–320 μg/ml) were required to obtain the same reduction in *S. mutans* and *S. sanguis* viability respectively. The minimal fungicidal concentration of MB for 5 log10 CFU reduction (4.5 McFarland) was 80–160 μg/ml, whereas for RB it ranged between 320 and 640 μg/ml. For CUR, even the maximum studied concentration (1280 μg/ml) did not reach that inhibition. Incubation time had no effect in all experiments.

Conclusions: Photodynamic therapy with RB, MB and CUR and white light is effective in killing *S. mutans* and *S. sanguis* strains, although MB and RB are more efficient than CUR. *C. albicans* required higher concentrations of all photosensitizers to obtain a fungicidal effect, being MB the most efficient and CUR ineffective.

Background

The human oral cavity is colonized by a highly diverse community of bacteria [1]. Dental caries is a chronic, invasive disease involving demineralization of the tooth followed by destruction of the organic phase of the dentine [2] and it is the consequence of the interaction between oral microflora, diet, dentition and oral environment [3].

Streptococci are the main colonizers of oral surfaces and constitute 70 % of the cultivable bacteria existing in the human dental plaque [4]. In fact, *S. mutans* is the most prevalent microorganism of the plaque and the primary pathogenic agent responsible for caries disease [5], whereas *S. sanguis* is thought to play a benign, if not a beneficial, role in the oral cavity [6]. On the other hand, *C. albicans* is a commensal fungal species commonly colonizing human mucosal surfaces [7]. Falsetta et al. [8] hypothesize that *S. mutans-C. albicans* association may enhance *S. mutans* infection and modulate the development of hypervirulent biofilms on tooth surfaces, which will in turn influence the onset and severity of dental caries *in vivo*. For this reason, *S. mutans*, *S. sanguis* and *C. albicans* should be included in any study about human dental plaque microorganisms.

Photodynamic therapy (PDT) has been advocated as an alternative to antimicrobial agents to suppress subgingival species [9] due to the extensive and inappropriate use of antimicrobial agents which gradually led to the development of pervasive resistance [10]. Antimicrobial PDT

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(aPDT) is a technique that utilizes reactive oxygen species (ROS) produced by non-toxic dye or photosensitizer (PS) molecules in the presence of low intensity visible light to kill mammalian or microbial cells [11]. Due to this mechanism, It is hypothesized that bacteria will not be easily able to develop resistance to PDT [12].

More than 400 compounds with photosensitizing properties are known, including dyes, drugs, chemicals and many natural substances [13]. Methylene blue (MB), a well-known dye with high light absorption at 665 nm, is effective in aPDT, showing ability to kill not only Gram positive and Gram negative bacteria but also fungi [14–17]. Rose Bengal (RB) is a xanthene dye characterized by light absorption at wavelengths (λ) of 450–600 nm, used for the diagnosis of eye diseases [18]. From an antimicrobial point of view, RB has shown a good profile to photoinactivate microorganisms [19–22]. Curcumin (CUR) is an intensely yellow pigment, isolated from rhizomes of *Curcuma longa*, with a peak of light absorption at 430 nm [23]. Among its many biological activities are its anti-carcinogenic, antioxidant, antiinflammatory, antimicrobial properties and its hypoglycemic effects in humans [24, 25]. Some studies have shown its capacity to effectively photoinactivate in vitro *C. albicans* [26, 27].

There are many papers exploring the aPDT effect of different photosensitizers (PSs) in almost all kind of microbial species [28–31]. However, only few of them compare the efficacy of several photosensitizers on different microorganism [32].

The aim of this study was to compare the photoinactivation effect of three PSs, MB, RB and CUR, on S. *mutans*, *S. sanguis* and *C. albicans*.

Results

Photoinactivation of bacterial suspensions

Under the experimental conditions, PDT with MB, RB and CUR inhibited 6 log10 the growth of both strains of bacteria reaching a bactericidal effect. However, less concentration of RB than of the other PSs was needed to kill *Streptococcus* spp. Whereas this bactericidal effect was achieved for *S. mutans* with a concentration of RB as low as 0.31–0.62 μg/ml, higher MB concentration (1.25–2.5 μg/ml) was needed to reach the same reduction. In the case of *S. sanguis*, the RB and MB concentrations needed to obtain the same bactericidal effect were quite similar to those used for *S.mutans* (0.62–1.25 μg/ml and 1.25–2.5 μg/ml, respectively). Much higher concentrations of CUR were necessary to obtain the same reduction either for *S. mutans* or *S. sanguis* (Table 1).

Regarding the effect of the incubation time of *Streptococcus* cells with the PSs, one hour halved the minimal concentration of MB or RB necessary to attain 6 log10 reduction respect to an incubation time lower than 1 min (<1 min), especially for *S. sanguis* (Table 1). In the case of CUR, this effect was only observed for *S. sanguis*. Not significant additional benefit was achieved using 3 h of incubation (Table 1).

Comparing the photodynamic effect of MB for *S. mutans* and *S. sanguis* suspensions, using the optimal incubation for each PS, lower concentrations were needed to reach the bactericidal effect for *S. sanguis* than for *S. mutans* (Fig. 1). However, no differences were observed using RB as PS.

Photoinactivation of *C. albicans*

Table 1 shows the minimum fungicidal concentration (MFC) of each PS starting from *C. albicans* 4.5 McFarland. Under the experimental conditions, PDT with MB, RB but not with CUR inhibited 5 log 10 the growth of *C. albicans*, being the needed concentrations of MB smaller than the RB ones (Fig. 2). An increase in the incubation time with the PS was only beneficial for MB, because 3 h halved the concentration needed to reach a 5 log10 reduction in *C. albicans* respect to shorter times (Table 1).

Discussion

Dental caries may be a disease well suited to PDT [2]. Our investigation showed that PDT using MB or RB and a white lamp can kill cariogenic microorganisms, such as *S. mutans*, *S. sanguis* and *C. albicans*. In contrast, even though PDT with CUR reaches the same bactericidal effect, much higher concentrations were needed and it was not effective against yeasts.

PDT efficacy depends on the microorganism, the PS and the light used. According to our results, RB showed higher antimicrobial photodynamic effect for

Table 1 Minimal range concentration to reduce 6 log10 of *S. mutans* and *S. sanguis* and 5 log10 of *C. albicans*

Pre-irradiation Incubation time (h)	MB	RB	CUR
S. mutans ATCC 35668			
<1 min	1.25–2.5 μg/ml	0.31–0.62 μg/ml	80–160 μg/ml
1 h	0.62–1.25 μg/ml	0.15–0.31 μg/ml	160–320 μg/ml
3 h	0.62–1.25 μg/ml	0.31–0.62 μg/ml	160–320 μg/ml
S. sanguis ATCC 10556			
<1 min	1.25–2.5 μg/ml	0.62–1.25 μg/ml	160–320 μg/ml
1 h	0.31–0.62 μg/ml	0.15–0.31 μg/ml	40–80 μg/ml
3 h	0.15–0.31 μg/ml	0.15–0.31 μg/ml	40–80 μg/ml
C. albicans ATCC 1023			
<1 min	80–160 μg/ml	320–640 μg/ml	>1280 μg/ml
1 h	80–160 μg/ml	>1280 μg/ml	>1280 μg/ml
3 h	40–80 μg/ml	>1280 μg/ml	>1280 μg/ml

Irradiation with metal halide lamp, λ 420–700 nm, fluence 37 $J.cm^{-2}$

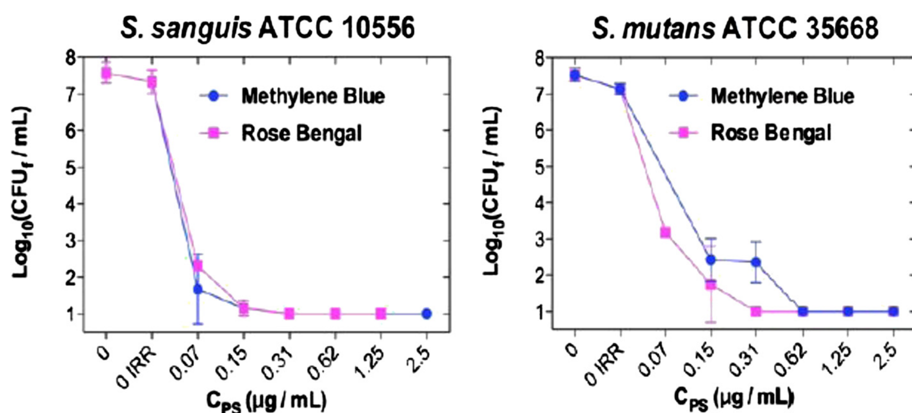

Fig. 1 Photodynamic effect of MB and RB on *S. sanguis* and *S. mutans* depending on their concentration (Incubation time with the PS <1 min and irradiation using a metal halide lamp with a fluence of 37 J/cm^2)

Streptococcus spp than the other PSs studied, whereas MB was better for *Candida* spp. CUR always showed the lowest antimicrobial activity; this could be due to the fact that the higher peaks of the spectrum emission of the lamp correspond better to the absorptium spectra of RB and MB than CUR. Table 2 shows that compared with previous studies using aPDT with MB, RB and CUR for *S. mutans*, our parameters seem to be more efficient, especially considering the percentage of bacterial growth inhibition of 99.9999 %. Regarding the antimicrobial effect of MB-PDT on the viability of *S. mutans*, Araujo et al. [33] needed 25 μg/ml MB to reach 73 % inhibition. Nevertheless, our results are not completely comparable because they used red light, which corresponds with the maximum spectrum absorbance of MB. Regarding RB, studies carried out by Costa et al. [34] show that the concentration needed to attain 6.86 log10 CFU/mL reduction of *S. mutans* was 2.02 μg/ml, using a LED lamp with λ 440–460 nm and a fluence of 95 J/cm^2. Comparing to our results they needed higher concentrations of PS to reach a similar effect, perhaps because the λ of their lamp was less convenient than ours to excite RB; another reason could be the use of distilled water as dissolvent, whereas they used phosphate-buffered saline because, based in the study of Nuñez et al. [35], a significant difference in the same aPDT experiment can be promoted only by the use of differents dissolvents.

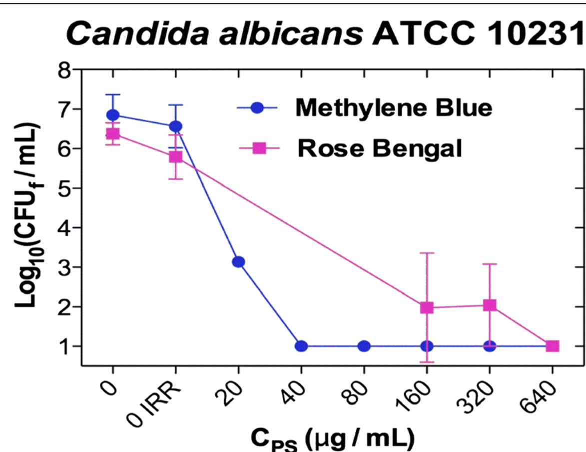

Fig. 2 Photodynamic effect of MB and RB on *C. albicans* depending on their concentration (incubation time of 1 h to MB and <1 min to RB, and irradiation using a metal halide lamp whit a fluence of 37 J/cm^2)

According to this study, CUR needs much higher concentration than RB and MB to photoinactivate bacteria. This result agrees with those obtained by Araujo et al. [23] who, using a concentration of 1500 μg/ml and blue LEDs lamp (λ 450 nm, fluence 5.7 J/cm^2), reached 60 % inhibition of *S. mutans* in planktonic cultures. However, other studies obtained 95 % reduction of *S. mutans* instead of 6 logs using only 0.73 μg/ml CUR, which could be explained by the higher fluence used (72 J/cm^2), the λ of the lamp (blue light) and the lower percentage of bactericidal activity obtained.

Few studies compare the efficacy of different PSs to photoinactivate oral microorganisms. Rolim et al. [36] showed that MB, toluidine blue ortho, malachite green, erythrosine, eosin and RB, using a red LEDs lamp for the former and a blue one for the later, were photoactive in vitro against *S. mutans,* but only toluidine blue reduced 99.9 % of the microorganism. In this study, they also find a bactericidal effect of RB on *S. mutans* without light. Nevertheless, in our study no antimicrobial effects were observed when the strains were exposed either to the dyes or the light source separately.

Considering that the cariogenic potential of *S. sanguis* is deemed low compared to that of the *S. mutans*, the number of reports using aPDT to kill *S. sanguis* is lower than those of *S. mutans*. Chan et al. [37] demonstrated

Table 2 Summary of the in vitro PDT studies using methylene blue, rose Bengal or curcumin on *S. mutans, S. sanguis and C. albicans*

	PS	Concentration (μg/ml)	Inhibition (%)	λ (nm)	Fluence (J/cm²)
S. mutans					
Araújo et al. [33]	MB	25	73	ND	ND
Our study	MB	2.5	999.999	420–700	37
Costa et al. [34]	RB	2.02	999.999	440–460	95
Our study	RB	0,62	999.999	420–700	37
Araújo et al. [23]	CUR	1500	99.9	450	5.7
Paschoal et al. [44]	CUR	1473.5	60	450	72
Manoil et al. [45]	CUR	0.73	95 %	360–550	542
Our study	CUR	160	999.999	420–700	37
S. sanguis					
Chan et al. [37]	AM	100	99–100	632.8	21.2
Our study	AM	2.5	999.999	420–700	37
Pereira et al. [32]	RB	5	9.9	455	95
Our study	RB	0.62	999.999	420–700	37
Our study	CUR	1500	999.999	420–700	37
Mattiello et al. [46]	TB	200	84.32	660	10
C. albicans					
Souza et al. [15]	AM	100	99.9	660	39,5
Peloi et al. [14] 4	AM	22,5	95,14	663	6
Souza et al. [16]	AM	100	88,6	685	28
Our study	AM	160	99.999	420–700	37
Costa et al. [18]	RB	23	9.9	455	95
Demidova et al. [47]	RB	200	99,9999	525–555	80
Our study	RB	>1280	<99.999	420–700	37
Andrade et al. [26]	CUR	7,3	89.5	455	5,28
Dovigo et al. [48]	CUR	14,8	85	440–460	18
Our study	CUR	>2460	<99.999	420–700	37

PS photosentizer, *MB* methylene blue, *RB* rose bengal, *CUR* curcumin, *TB* toluidine blue ortho, *ND* no data

that PDT with MB was able to obtain a reduction of 99–100 % on cultures of *S. sanguis*. However, they used higher concentrations (100 μg/ml) than we used but lower fluence (21.2 J/cm²) of a diode laser (665 nm). Pereira et al. [32] only obtained a 9.9 % inhibition with 5 μg/ml RB and they used a higher fluence. Therefore, our results show MB as the most efficient bactericidal PS for *S. sanguis*.

According to the present investigation, MB has better antifungal profile than RB and CUR. Other authors [14, 15, 38], show that MB–aPDT is endowed with antifungal potential against *C. albicans,* whereas Costa et al. [18] show that RB only reach a 1,97 log10 reduction in *C. albicans*.

Comparing the three PSs, the present study shows that MB is the most effective PS on *C. albicans* while RB was slightly superior for *S. mutans*. Dental caries result from interactions among different cariogenic microorganisms, so using or combining different PSs could improve the efficacy of aPDT. In this sense, the use of a white light lamp that covers all the absorption spectrum of most PSs can efficiently excite them, making PDT easier to perform and avoiding the necessity of using a lamp for each PS. However, a light source with an emission spectrum that corresponds to the maximum absorption spectrum of each PS theoretically determines a higher efficacy [39]. Red light sources (630–700 nm) have been used extensively in PDT due to their relatively long wavelengths, which can effectively penetrate biological tissues and activates some of the most effective PSs, such as phenothiazines and porphyrins. Additionally, other studies have also shown that blue light (380–520 nm) is an attractive option for PDT, because blue light sources can be used in combination with many PSs, such as RB, eosin, erythrosine, and CUR to photoinactivate oral microorganisms [36]. For this reason, one limitation of our study is the use of the same lamp to photoactivate the three PSs, whose emission spectrum matches quite accurately with the maximum absorptium spectra of RB and MB but not with CUR. This could influence the bad results obtained with the later.

According to our data, the minimal bactericidal or fungicidal concentration was reduced in some experiments with a pre-irradiation incubation time of 1 h. However, considering that the increase in the concentration was only of one or two dilutions, the difficulty to maintain therapeutic concentrations of the PS in the high flow conditions within the oral cavity (due to saliva and/or gingival crevicular fluid) for a long period of time [40] do not support the use of incubation time in the clinical setting. Andrade et al. [26] using CUR and Rezusta et al. [41] with hypericin concluded that none incubation time enhance the photoinactivation of planktonic cultures of *C. albicans*. Additionally, although the adverse effects of blood and saliva could be avoided with the help of dental dams, not pre-incubation time is more comfortable for the patients and more efficient for doctors.

Conclusions

The photodynamic efficacy of each PS varies according to the target microorganism. The combination of different PS and white light could be a promising approach to treat those infections caused by a combination of microorganisms, such as caries.

Materials and methods

Chemicals

Methylene Blue (MB) and Curcumin (CUR) were purchased from Sigma-Aldrich and Rose Bengal from Fluka. Sabouraud Dextrose Agar (SB) and Columbia Blood Agar (BA) were purchased from Oxoid.

Microorganisms and growth conditions

S. mutans ATCC 35668, *S. sanguis* ATCC 10556 and *C. albicans* ATCC 10231 strains were obtained from the American Type Culture Collection (ATCC; Rockville, MD).

McFarland scale is recommended for performance of susceptibility testing by CLSI [42] and EUCAST [43].

The yeasts were grown aerobically overnight in SB medium at 35 °C. Stock inoculum suspensions were prepared in distilled water and adjusted to optical densities corresponding to 4.5 McFarland for five logs reduction assays. Cell viability was assessed counting the number of colony-forming units (CFU), developed on SB after an incubation period of 24 h at 35 °C.

S. mutans and S. sanguis were grown aerobically in BA medium at 35 °C for 48 h. Stock inoculum suspensions were prepared in distilled water and adjusted to optical densities corresponding to 0.5 McFarland for six logs reduction assays. Cell viability was assessed counting the number of CFU, developed on BA after an incubation period of 48 h for *S. mutans* and 24 h for *S. sanguis* at 35 °C.

Photosensitizer solutions

Stock MB, RB and CUR solutions were prepared in distilled water and diluted either with bidistilled water to the desired concentration immediately prior to use. The concentrations used ranged from 0.1–12800 μg/ml. All solutions were prepared and handled under light-restricted conditions.

Light source

In order to cover the spectrum absorption of the 3 PSs (Fig. 3), MB, RB and CUR (maximal absorption λ at 665, 557 and 430 nm respectively), we used a metal halide lamp emitting at 420–700 nm (Fig. 4) with an irradiance of 90 mW/cm^2, being the specific irradiance at the maximal absorptium λ of each PS : 292 μW/cm^2 at 557 nm, 300 μW/cm^2 at 665 nm and 186 μW /cm^2 at 430 nm.

Microorganisms suspensions with the different PSs prepared into a 96 wells microtiter plate, with a well diameter of 6 mm, were irradiated for 6 min and 51 s at a distance of 10 cm. The light beam diameter was 21 cm and the

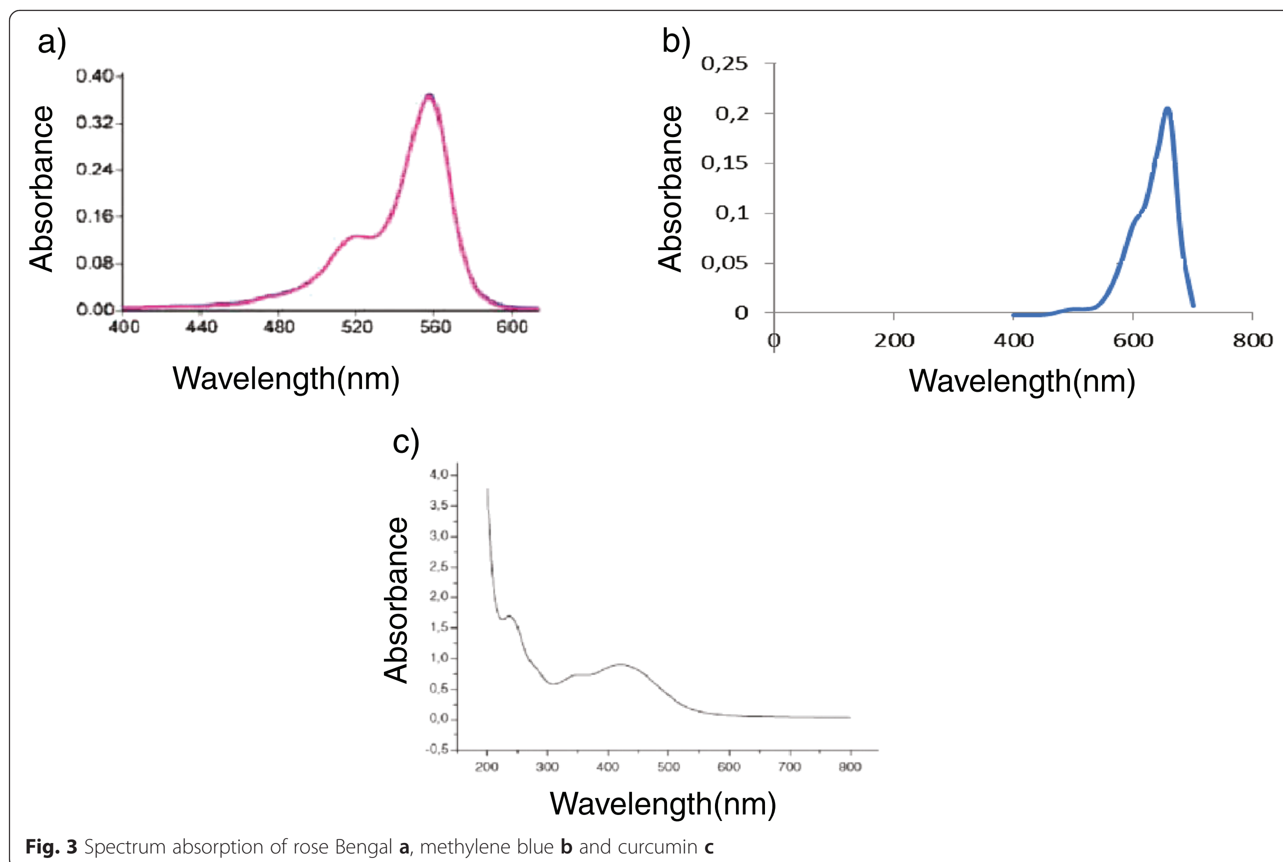

Fig. 3 Spectrum absorption of rose Bengal **a**, methylene blue **b** and curcumin **c**

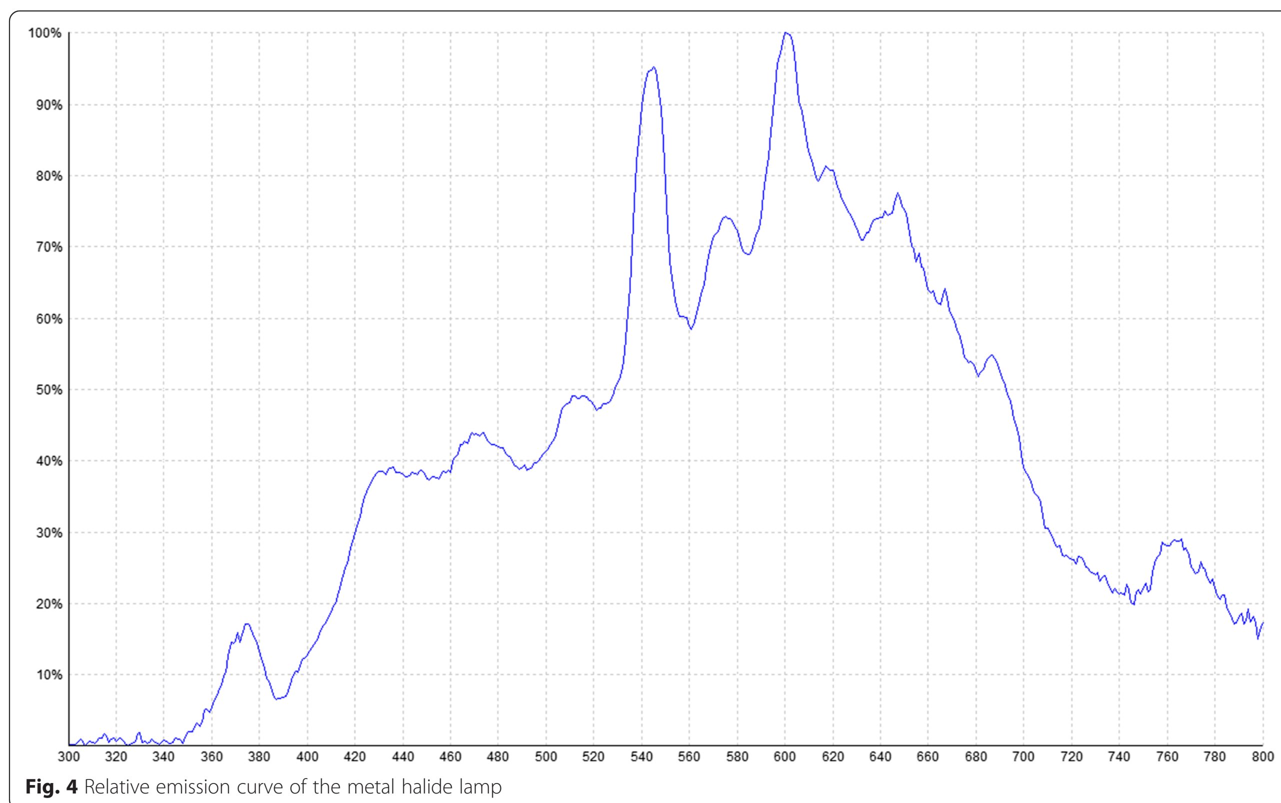

Fig. 4 Relative emission curve of the metal halide lamp

fluence used 37 J/cm^2. The effective light received on the dishes results from integrating the power of the lamp for all the effective wavelengths for each PS.

Photodynamic treatments of microorganisms

Suspensions with the desired McFarland value of every microorganism were prepared in bidistilled water. 90 μL of these initial suspensions was dropped into different wells of a microtiter plate and 10 μL of the different PS solutions were added. The final PS concentration in the experiments used ranged from 0.01–1280 μg/ml. The plates were then maintained in the dark for different periods of time (<1 min, 1 and 3 h) to evaluate the influence of contact time with the PS on the outcome of the photodynamic treatments. Afterwards, microorganisms were subjected to illumination (37 J/cm^2).

Fungal and bacterial cultures grown under the same conditions with and without PS, either kept in the dark or illuminated, served as controls.

After photodynamic treatments, samples and controls were incubated at 35 °C for 24 h, in case of *C. albicans* and *S. sanguis* experiments, and for 48 h in case of *S. mutans*. The antimicrobial effect was determined by counting the number of CFU per millilitre.

A criterion of 5 log10 unit decrease from the starting inoculum was adopted to define fungicidal activity, and a more stringent criterion of 6 log10 unit for bactericidal activity, due to the differences in cell size and mass between *Candida* spp and *Streptococcus* spp (the cell concentration of *C. albicans* used was 10 times lower (>10^5) than that used for the two bacterial species (>10^6)).

All experiments were carried out at least five times.

Abbreviations

MB: Methylene blue; RB: Rose bengal; CUR: Curcumin; CFU: Colony forming units; PDT: Photodynamic therapy; aPDT: Antimicrobial photodynamic therapy; PS: Photosensitizer; SB: Sabouraud dextrose agar; BA: Columbia blood agar.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PSL conceived of the study, conducted all experiments and drafted the manuscript. YG participated in the design of the study, in the interpretation of data for the work and she drafted the manuscript, the cover letter and the answers to the reviewer's reports. LPA and VLP conducted some experiments and helped to draft the manuscript. VPL is studying the photodynamic effect in co-cultures of *C. albicans* and *S. mutans* and she checked our results. She also revised the manuscript and contributed to the resolution of reviewer's reports. IGL has contributed to the interpretation of data for the work and to the resolution of biofilms questions and in the discussion of the final manuscript because she works with us in the project CTQ 2013–48767-C3-2-R from the Spanish Ministry of Science and Innovation studing the effect of photodynamic therapy on biofilms. MPPC participated in the design of the study, analyzed the data, performed the Figs. 1 and 2 and he revised the manuscript particularly the methodology and the materials. JAA performed the Figs. 3 and 4 and he wrote everything related light parameters both in the manuscript and in the answers to the reviewer's reports. MJR contributed to the design of the work, and revising and correcting it, particularly the background and the discussion. AR conceived of the study and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This study was supported by grants CTQ2013-48767-C3-2-R from the Spanish Ministry of Science and Innovation, Spain and European Regional Development Fund.

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Received: 6 November 2014 Accepted: 21 September 2015
Published online: 26 September 2015

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